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(54) Title: PHARMACEUTICAL LIPID AGGREGATE WITH HELICOBACTER PYLORI ANTIGEN AND NEGATIVELY CHARGED LIPID

(57) **Abstract**

The present invention relates to a new pharmaceutical formulation comprising a lipid aggregate of a negatively charged lipid or lipid mixture and at least one antigenic, native or recombinant polypeptide which constitute a *Helicobacter pylori* antigen, and optionally a pharmaceutically acceptable carrier. More specifically the invention relates to a lipid aggregate comprising a surface-exposed antigen with an approximate molecular weight of 29 kDa. Furthermore the invention provides a formulation of a nucleic acid molecule coding for the said polypeptide. The new formulations, which are useful as vaccine formulations, elicit a protective immune response against *H. pylori* infections, and said vaccine formulations are suitable for both therapeutic and prophylatic use.

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PHARMACEUTICAL LIPID AGGREGATE WITH HELICOBACTER PYLORI ANTIGEN AND NEGATIVELY CHARGED LIPID

Field of the invention

The present invention provides a new pharmaceutical formulation comprising polypeptides, which constitute *Helicobacter pylori* antigens, or nucleic acid molecules coding for the said polypeptides. More specifically the present invention relates to a pharmaceutical formulation in the form of a lipid aggregate comprising these polypeptides or antigens. Furthermore, the invention provides a process for the preparation of such a pharmaceutical formulation, and the use of such a formulation in the treatment of *Helicobacter pylori* infections, as well as a method of treating a patient to elicit a protective immune response against *Helicobacter pylori* infections, said formulation being suitable for both therapeutic and prophylactic use.

Background of the invention

There is a need for an effective drug delivery system, such as a vaccine composition, for polypeptides or antigens involved in the protection and treatment of *H. pylori* infections. The pharmaceutical formulation shall provide an improved immune response to the polypeptides or antigens delivered by the formulation.

The gram-negative bacterium *Helicobacter pylori* is an important human pathogen, involved in several gastroduodenal diseases. Colonization of gastric epithelium by the bacterium leads to active inflammation and progressive chronic gastritis, with a greatly enhanced risk of progression to peptic ulcer disease.

In order to colonize the gastric mucosa, *H. pylori* uses a number of virulence factors. Such virulence factors comprise several adhesins, with which the bacterium associates with the mucus and/or binds to epithelial cells; ureases which helps to neutralize the acid environment; and proteolytic enzymes which makes the mucus more fluid.

Despite a strong apparent host immune response to *H. pylori*, with production of both local (mucosal) as well as systemic antibodies, the pathogen persists in the gastric mucosa, normally for the life of the host. The reason for this is probably that the spontaneously induced immune-response is inadequate or directed towards the wrong epitopes of the antigens.

In order to understand the pathogenesis and immunology of *H. pylori* infections, it is of great importance to define the antigenic structure of this bacterium. In particular, there is a need for characterization of surface-exposed (like adhesins) and secreted proteins which, in many bacterial pathogens, have been shown to constitute the main virulence factors, and which can be useful for the diagnosis of *H. Pylori* and in the manufacture of vaccine compositions.

Cloning of the *hpaA* gene, which codes for a 20 kDa receptor-binding subunit of the *N*-acetylneuraminyllactose-binding fibrillar hemagglutinin (NLBH) of *H. pylori*, has been disclosed by Evans et al. (1993) J. Bacteriol. 175, 674-683.

Monoclonal antibodies (MAbs) against membrane preparations of *H. pylori* have been disclosed by Bölin et al. (1995) J. Clin. Microbiol. 33, 381-384. One of these MAbs, designated HP30-1:1:6, reacted with a 30 kDa protein which was shown to be exposed on the surface of intact bacteria and to have properties like that of an adhesin.

Whenever stressed or threatened, the *H. pylori* cell transforms from a bacillary to a coccoid form. In the coccoid form, the *H. pylori* cell is much less sensitive to antibiotics and other anti-bacterial agents. Circumstantial evidence indicate the *H. pylori* might be transmitted between individuals in this form, possibly via water or direct contact. An efficient vaccine composition should therefore elicit an immune response towards both the coccoid and the bacillary form of *H. pylori*. Since systemic immunity probably only plays a limited role in protection against mucosal infections,

it is also important that the vaccine composition will enhance protective immune mechanisms locally in the stomach.

Prior Art

Several different types of vaccine formulations have been described in the literature (see e g "Vaccine Design. The subunit and adjuvant approach" (Eds: M F Powell and M J Newman), Pharmaceutical Biotechnology, vol 6, Plenum Press, NY 1995). Thus, vaccines have been formulated in known delivery systems such as liposomes, ISCOMs, cochleates, etc, or have been attached to or included into polymer microspheres of degradable or non-degradable nature. Antigens have been associated with live attenuated bacteria, viruses or phages or with killed vectors of the same kind. Other acceptable carriers or diluents, known to those skilled in the art, are e g phosphate buffered saline, enterically coated powder formulations, surface active substances and polymers, etc.

Specifically, aggregates formed between amphiphilic molecules, e g phospholipids, and ions of opposite charge, have been studied extensively (e g Nir et al, Progress in Surface Science, 13 (1983), 1 - 124) and also sometimes been reported as suitable formulations or formulation components for different types of therapeutic agents, including antigens. In some of these reports the focus have been on the use of negatively charged phospholipids, e g phoshatidylserine, phosphatidylglycerol, etc, and positively charged ions like Ca^{2+}, Mg^{2+}, etc. Large, cylindrical structures with lipid multilayers in a spiral configuration was reported to exist in mixtures of phosphatidylserine and Ca^{2+} in water by Papahadjopoulos et al (*Biochim Biophys Acta* 394, 483-491 (1975)) and named cochleates by the authors. The morphology of these structures as well as prerequisites and techniques for their formation, either in pure phospholipid systems, or in combination with other substances, e g other phospholipids, cholesterol, polypeptides like antigens, DNA etc, have been reported in a number of studies (e g Galla and Sackmann, *Biochim Biophys Acta* 401, 509-529 (1975); Papahadjopolous et al. *Biochim Biophys Acta* 465, 579-598 (1977); Wilschut and

Papahadjopoulus *Nature* 281, 690-692 (1979); Tilcock et al. *Biochemistry* 23, 2696-2703 (1984); Silvius and Gagné *Biochemistry* 23, 3241-3247 (1984); Graham et al. *Biochemistry* 24, 7123-7131 (1984); Gould-Fogerite and Mannino *Analytical Biochemistry* 148, 15-25 (1985) Kouaouci et al. *Biochemistry* 24, 7132-7140 (1985); Gould-Fogerite et al. in *Advances in Membrane Biochemistry and Bioenergetics* (Eds: Kim et al.), Plenum Press NY 1988, pp 569-586; Mannino and Gould-Fogerite *Biotechniques* 6, 682-690 (1988); Gould-Fogerite et al. *Gene* 84, 429-438 (1989); Roux and Bloom *Biophys J* 60, 38-44 (1991); Flach and Mendelsohn *Biophys J* 64, 1113-1121 (1993); Gould-Fogerite and Mannino in *Liposome Technology* (Ed Gregoriadis), CRC Press, Boca Raton 1993, vol I pp 67-80, and vol III pp 261-276; Nieva et al. *Biochemistry* 33, 3201-3209 (1994); Hinderliter et al. *Biophys J* 67, 1906-1911 (1994)). A few patents and patent applications have also described drug delivery systems based on either cochleate structures directly, or on systems derived from cochleate structures (Papahadjopoulos US78/4078052; Mannino and Gould-Fogerite US87/4663161; Mannino and Gould-Fogerite US89/4871488; Gould-Fogerite and Mannino WO95/09648).

Purpose of the invention

The purpose of this invention is to provide a new pharmaceutical formulation of an antigenic *Helicobacter pylori* polypeptide or a pharmaceutical formulation of a nucleic acid molecule coding for such a polypeptide. More specifically, the present invention relates to a new pharmaceutical formulation based on a lipid aggregate of a negatively charged lipid or mixture of lipids, and at least one polypeptide which is a Helicobacter pylori antigen, or nucleic acid molecules coding for such polypeptides, and optionally a pharmaceutically acceptable carrier. One suitable antigen for the lipid aggregate is a 29 kDa polypeptide, obtained by the recombinant cloning of a *H. pylori* gene which encodes a surface exposed protein. The 29 kDa polypeptide is described below as well as in the Applicant's co-pending patent application WO96/38475, published 5 December 1996. The nucleic acid sequence of the 29 kDa

polypeptide is similar to the sequence of the *hpaA* gene as published by Evans et al. (1993) in Journal of Bacteriology, vol 175, 674-683. Thus, the pharmaceutical formulation according to the present invention is also suitable for the *hpaA* gene.

In general terms, the new pharmaceutical formulation is also suitable for other *H. pylori* antigens, especially membrane proteins. In the following such suitable antigens for the formulation are the 29 kDa polypeptide described below, or the *hpaA* gene described by Evans et al. The different names 29 kDa and *hpaA* gene or HpaA protein are used interchangable in the present specification as suitable antigens for the present formulation, even if the sequence of the *hpaA* gene from different *H. pylori* strains shows some differences (P.W. Toole et al, Bacteriology Vol. 177, No. 21, Nov. 1995).

The 29 kDa polypeptide is described in the Applicant's co-pending WO96/38475, published 5 December 1996, as an antigenic protein which is expressed in all strains of *H. pylori*, also in coccoid forms of the bacterium, and which is able to induce a mucosal as well as a systemic immune-response in a host measured as antibody production. The 29 kDa polypeptide is expressed by all *H. pylori* strains tested, and antibodies created towards this protein do not cross-react with common endogenous human bacteria of other species or with selected human tissues including the gastric mucosa. Thus being an essential, well conserved adhesin with immunogenic properties, the 29 kDa polypeptide is useful both for the detection of *H. pylori* infections as well as for the manufacture of vaccine compositions. When given in an appropriate pharmaceutical formulation, as the new formulation according to the present invention, the 29 kDa polypeptide elicits a protective or therapeutic immune response against such *H. pylori* infections.

Description of the 29 kDa polypeptide

The 29 kDa polypeptide is described in WO96/38475 hereby incorporated in whole by reference. It has an amino sequence substantially similar to a *Helicobacter pylori*

surface-exposed antigen and has an approximate molecular weight of 29 kDa. The said surface-exposed antigen has i.a. the following important properties:

- It is an adhesin, which is important for the colonization of the gastric mucosa:
- It is expressed on the surface of both dividing (bacillary) forms as well as resting (coccoid) forms of *H. pylori*;
- It is a strong antigen giving rise to both systemic and local (mucosal) production of antibodies;
- It is conserved in all tested strains of *H. pylori*;
- Antibodies to the 29 kDa polypeptide do not cross-react with a number of different non-helicobacter bacteria, or with selected human tissues, including the gastric mucosa;
- The 29 kDa polypeptide is lipidated and thus post-translationally modified. This feature of the polypeptide may be of importance for its immunogenicity and for its proper exposure on the surface of *H. pylori*. It is known in the art that lipid modification can be essential for the immunological properties of bacterial lipoproteins (see Weis, J.J. et al. (1994) Infection and Immunity, vol. 62, 4632-4636).
- It is a putative virulence-factor, whereby the term "virulence factor" is to be understood a molecule specifically involved in adherence of *H. pylori* to the epithelial surface of the gastric mucosa and / or in the establishment and maintenance of *H. pylori* infection.

In a preferred form, the said polypeptide has an amino acid sequence according to positions 1-260, or 28-260, in SEQ ID NO: 2 or 4 of the Sequence Listing. Further, it is believed that positions 1-260 in SEQ ID NO: 2 and 4 represent the uncleaved protein, while positions 1-27 represent a signal sequence and positions 28-260 represent the mature polypeptide. The only difference between SEQ ID NO: 2 and SEQ ID NO: 4 is that SEQ ID NO: 2 has a Ser residue in position 222, while SEQ ID NO: 4 has an Arg residue in the same position.

However, the polypeptides which can be formulated according to the invention is not to be limited strictly to the 29 kDa polypeptide with an amino acid sequence identical with the above mentioned positions in SEQ ID NO: 2 or 4 in the Sequence Listing. Rather the invention encompasses polypeptides carrying modifications like substitutions, small deletions, insertions or inversions, which polypeptides nevertheless have substantially the properties of the 29 kDa polypeptide. Such properties include the ability to elicit a mucosal as well as systemic immune-response against *H. pylori* in a mammal host; the ability to work as an adhesin; and the presence of the polypeptide in both bacillary and coccoid forms of *H. pylori*.

Consequently, one purpose of the invention is to present a formulation based on a lipid aggegate of polypeptides, the amino acid sequence of which is at least 90% homologous, preferably at least 95% homologous, with the amino acid sequence shown as positions 1-260, or positions 28-260, in SEQ ID NO: 2 or 4, in the Sequence Listing, which polypeptides nevertheless have substantially the biological activities of the 29 kDa polypeptide.

Further, the purpose of the invention is to provide a formulation based on a lipid aggregate of peptides, with a length of at least 5 amino acids, which comprise an immunogenic epitope of the 29 kDa polypeptide and retains the ability to elicit an immune response against *H. pylori* bacteria in a mammal host. Such epitope(s) can be presented alone or in the form of fusion proteins, where the epitope is fused to an inert or immunologically active carrier polypeptide. The identification of these epitopes will be based on the presence of host-generated antibodies towards different segments of the 29 kDa polypeptide.

One way of obtaining structural information on the epitopes of the 29 kDa polypeptide is the production and characterisation of monoclonal antibodies binding to the polypeptide, followed by mapping of epitopes by e.g. Pepscan analysis.

Monoclonal antibodies can be produced by standard methods, such as those described by De St. Groth (1980) in J. Immunol. Methods, vol. 35, 1-21.

In another aspect, the invention provides a formulation based on a lipid aggregate of an isolated and purified nucleic acid molecule which has a nucleotide sequence coding for a polypeptide as defined above. In a preferred form of the invention, the said nucleic acid molecule is a DNA molecule which has a nucleotide sequence identical with SEQ ID NO: 1 or 3 of the Sequence Listing. However, the DNA molecule is not to be limited strictly to the sequence shown as SEQ ID NO: 1 or 3. Rather the invention encompasses formulations of DNA molecules carrying modifications like substitutions, small deletions, insertions or inversions, which nevertheless encode polypeptides having substantially the biochemical activity of the 29 kDa polypeptide. It will be known to the skilled person that A ↔ G and T ↔ C substitutions, with no effect on the amino acid sequence, are not unusual in *H. pylori*. The only difference between SEQ ID NO: 1 and SEQ ID NO: 3 is that SEQ ID NO: 1 has an A residue in position 1458, while SEQ ID NO: 3 has a C residue in the same position.

Furthermore, the invention provides a formulation of DNA molecules which nucleotide sequences are degenerate, because of the genetic code, to the nucleotide sequence shown as SEQ ID NO: 1 or 3. Since there are 64 possible codons, but only 20 natural amino acids, most amino acids are coded for by more than one codon. This natural "degeneracy", or "redundancy", of the genetic code is well known in the art. It will thus be appreciated that the DNA sequence shown in the Sequence Listing is only an example within a large but definite group of DNA sequences which will encode the polypeptide as described above.

Consequently, the inventions includes also formulations based on a lipid aggregate of an isolated nucleic acid molecule selected from:

(a) nucleic acid molecules comprising a nucleotide sequence which is identical with, or substantially similar to, positions 796-1572 or 874-1572 in SEQ ID NO: 1 or 3 in the Sequence Listing;

(b) nucleic acid molecules comprising a nucleotide sequence capable of hybridizing to a nucleotide sequence complementary the polypeptide coding region of a DNA molecule as defined in (a) and which codes for a polypeptide as described above, or a functionally equivalent modified form thereof; and (c) nucleic acid molecules comprising a nucleic acid sequence which is degenerate as a result of the genetic code to a nucleotide sequence as defined in (a) or (b) and which codes for a polypeptide as described above, or a functionally equivalent modified form thereof.

Brief description of the drawings

Figure 1a) shows the result of eradication of *H. pylori* infection in mice after oral administration of different agents, such as a *Helicobacter* antigen alone or together with an adjuvant; the antigen is administered either directly (control) or incorporated in lipid aggregates according to the present invention. Legend to figure 1a) and abbrevations are as follow:

Number of *H. pylori* (as CFU, geometric mean values) in 25mm^2 scrapings of antrum and corpus, following oral administration of different agents.

CFU= Colony forming units; CT=Cholera toxin; LA=Lipid aggregates; LA(29 kDa)=29 kDa polypeptide formulated in Lipid aggregates.

Figure 1b) shows the same as Figure 1a) but displayed as the total number of *H. pylori* in the stomach. Abbrevations see: Figure 1a) above.

Figure 2) shows mucosal antibody response towards a *Helicobacter* antigen incorporated in a lipid aggregate according to the present invention. The figure 2) shows specifically the total amount of mucosal immunoglobulin (Ig) with a specificity for the antigen preparation

comprising the 29 kDa polypeptide, detected by ELISA technique. Abbrevations see: Figure 1a) above.

Figure 3) shows mucosal antibody response towards a *Helicobacter* antigen incorporated in a lipid aggregate according to the present invention. The figure 3) shows specifically the amount of mucosal IgA antibodies with a specificity for the antigen preparation comprising the 29 kDa polypeptide. Abbrevations see: Figure 1a) above.

Figure 4) shows serum antibody response towards a *Helicobacter* antigen incorporated in a lipid aggregate according to the present invention. The figure 4) shows specifically the amount of Helicobacter specific IgG antibodies in sera with a specificity for an antigen preparation comprising a membrane protein. Abbrevations see: Figure1a) above.

Figure 5) shows serum antibody response towards a *Helicobacter* antigen incorporated in a lipid aggregate according to the present invention. The figure 4) shows specifically the amount of 29 kDa specific IgG antibodies in sera with a specificity for an antigen preparation comprising 29 kDa polypeptide. Abbrevations see: Figure 1a) above.

Disclosure of the invention

It has now been found that an improved immune response to *Helicobacter pylori* antigens as defined above can be obtained by administration of said antigens in a pharmaceutical formulation based on lipidic material, i.e. in the form of a lipid aggregate.

Means of obtaining the improved formulations are based on the use of negatively charged lipids, either alone or in combination with other lipid material such as, but not limited to phospholipids, glycerides, etc, and in combination with a cation component, such as positively charged ions. The use of the formulations result in an improved immune response to the antigens described above when given by different

administration routes, such as, but not limited to, the oral, the rectal, the tonsillar, the buccal, the nasal, the vaginal etc, administration route. The preferred administrations are the peroral, rectal and nasal routes.

The negatively charged lipids can be, but are not limited to: phosphatidylserine, phosphatidylinositol, phosphatidic acid, and phosphatidylglycerol.

The said negatively charged lipids might be combined with one or more additives in the formulation, such as, but not limited to:

- *other lipids*, e g cholesterol, phosphatidylcholine, phosphatidyletanolamine, etc;
- *ions*, e g Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Mn^{2+}, Al^{3+} etc.
- *adjuvants to further enhance the immunological response*, e g lipid A and its derivatives, saponins like QS-21, cholera toxin (CT), etc.

The process chosen for manufacturing of said pharmaceutical formulation is exemplified by the following general description, and in the Experimental Section below.

In general, these procedures are based on the formation of the lipid aggregates in three steps:

- the formation of a lipid film composed of the negatively charged lipids with or without other lipids, e g cholesterol;
- the addition of the antigen in a surfactant solution which then also produces mixed micelles with the lipids with incorporated antigen, preferably a nonionic surfactant with high critical micelle concentration is used;
- the exchange of the surfactant with the positively charged ion.

The last step can be carried out in different ways. One way is to first remove the surfactant, e g by dialysis, and then add for example Ca^{2+} in a second dialysis step, or

directly from a concentrated Ca^{2+} solution. Another way is to exchange the surfactant with the positively charged ion in one dialysis step. A modification of these processes can be utilized if the antigen is insensitive to organic solvents. Thus, the antigen is dissolved together with the lipids in an organic solvent. The formation of liposomes with incorporated antigen is then formed after evaporation of the solvent and after the dispersion in a buffer solution. The lipid aggregates can finally be induced by the addition of positively charged ions by direct addition or by dialysis. Modifications of these processes are possible and can be chosen according to literature by those skilled in the art.

The dosage form used may be a solid, semisolid, liquid dispersion, or solutions prepared by use of well known pharmaceutical techniques, such as blending, granulation, milling, spray drying, compaction, coating, etc. Further, the formulations may be monolithic, such as tablets, or capsules, or in the form of multiple formulations administered in a tablet, capsule or sachets.

The antigen is a polypeptide for use in therapy of *Helicobacter pylori* infection in a mammal, including man, and for use as a therapeutic or prophylactic vaccine as described above.

Another important aspect of the invention is a vaccine formulation for inducing a protective immune response in a mammal, including humans, against the bacillary and/or coccoid form of *Helicobacter pylori*. Such a vaccine formulation comprises a lipid aggregate and an immunogenically effective amount of a polypeptide as defined above. Preferably the polypeptide includes at least a part of the 29 kDa polypeptide comprising an immunogenic epitope, or a modified form of said polypeptide which retains the capability to induce protective immunity against *Helicobacter pylori* infection. The term "modified form" includes, but is not restricted to, forms of the polypeptide which are post-translationally modified, e.g. lipidated. It is believed that the 29 kDa protein is lipidated.

The vaccine formulation according to the invention can be used for both therapeutic and prophylactic purposes. The vaccine formulation according to the invention is preferably administered to any mammalian mucosa exemplified by the buccal, the nasal, the tonsillar, the gastric, the intestinal (small and large intestine), the rectal and the vaginal mucosa. The mucosal vaccines can be given together with for the purpose appropriate adjuvants. The vaccine can also be given parenterally, by subcutaneous, intracutaneous or intramuscular route, optionally together with the appropriate adjuvant. With the term "adjuvant" is meant a component which in general enhances the effect of the vaccine. Such adjuvants are for instance cholera toxin and E.coli heat-labile toxin and their non-toxic derivatives saponins, cytokines and chemokines. Non-toxic derivatives of cholera toxin and E.coli heat labile toxin includes e.g. mutations with reduced ADP-ribosylating activity or CTA1-DD which binds only to $1g^+$ cells.

An alternative approach for creating an immune response against the 29 kDa polypeptide is to use the approach known as "nucleic acid vaccination" or "naked DNA" vaccination. It is known in the art that injection into muscle of plasmid DNA encoding an antigen of interest can result in sustained expression of the antigen and generation of an immune response (see e.g. Rabinovich et al. supra). Several routes of administration are possible, such as parental, mucosal or via a "gene-gun" that delivers tiny amounts of DNA-coated gold beads (Fynan et al. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 11478-11482).

Thus, a nucleic acid molecule can be expressed in plasmid comprising a suitable eukaryotic promoter. This "naked DNA" can then be given, e g orally with the formulation. Epitopes of the expressed protein will be expressed by MHC molecules on the surface of the cells and trigger an immune response. Consequently, nucleic acid molecules and vectors as disclosed in the previous paragraphs for use in therapy, in particular for use as a vaccine, are further aspects of the invention. The

use of such nucleic acid molecules and vectors in the manufacture of compositions for treatment, prophylaxis or diagnosis of Helicobacter pylori infection are also further aspects of the invention.

In a further aspect, the invention provides a method of eliciting in a mammal, including man, a protective immune response against *Helicobacter pylori* infection, said method comprising the step of administering to the said mammal an immunologically effective amount of a vaccine formulation according to the present invention. The term "immunologically effective amount" is intended to mean an amount which elicit a significant protective *Helicobacter pylori* response, which will eradicate a *H. pylori* infection in an infected mammal or prevent the infection in a susceptible mammal. Typically an immunologically effective amount would comprise approximately 1 µg to 1 000 mg, preferably approximately 100 µg to 100 mg, of a *H. pylori* antigen for oral administration, or approximately less than 100 µg for parenteral administration. An immunologically effective amount of the antigen is typically administered in a formulation with the antigen in a range from 0.01 % w/w to 99 % w/w.

Detailed description of the invention

The following examples illustrative different aspects of the invention. The preparation of the lipid aggregate comprising a *H. pylori* antigen is described in Example 1 and its use is illustrated in Example 2. Examples 3 to 7 describe the preparation and tests of a suitable antigen for the pharmaceutical formulation prepared in Example 1, i.e. an antigen for the formulation according to the present invention.

Preparation and testing of a formulation according to the present invention

EXAMPLE 1: Formulation of the 29 kDa polypeptide rHpaA

1.1 Materials & Methods

Materials: BPS (brain phosphatidylserine, Avanti), CH (cholesterol), TES (N-tris(Hydroxymethyl)methyl-2-aminoethanesulfonic acid), L-histidine, NaCl, NOG (N-octyl-β-D-glucopyranoside), $CaCl_2$ $2H_2O$, NaOH, chloroform, and methanol were used as purchased. The water was ELGA quality (18.2 MΩ). The 29 kDa polypeptide and polyclonal rabbit-anti-29 kDa were obtained inhouse according to the examples described below.

Methods: In this specific example, the final composition of the formulation was intended to contain 1 mg/mL of the antigen, with a lipid/antigen weight ratio of 4/1, and with a total lipid concentration in the range of 2.5 - 3 mg/mL. Thus, a lipid film of BPS and CH (ratio 9/1 w/w) was obtained in a rotavapor system by evaporation of the chloroform/methanol (9/1 v/v) solvent that had been used for mixing the lipids. The lipid film was dissolved in a buffer solution (2 mM TES, 2 mM L-histidine, 100 mM NaCl, pH adjusted to 8.0 with NaOH(aq)) containing 2% w/w NOG (the NOG/lipid ratio intended to be 7.5/1 w/w) with or without the antigen. NOG was exchanged for Ca^{2+} during dialysis (Spectrapoor no 1, MW cutoff 6000-8000), twice versus a 3mM Ca^{2+} buffer solution (overnight and four hours), and twice versus a 6 mM Ca^{2+} solution (overnight and four hours).

After centrifugation of the contents, the supernatant and the pellet were analyzed for their antigen contents by different methods (Lowry assay, ELISA (polyclonal rabbit-anti-29 kDa), SDS-PAGE, and Western blot). The following results were obtained:

- By Lowry: concentration in the total formulation: 0.41 ± 0.05 mg/mL; pellet: 0.41 ±0.04 mg/mL. In the supernatant as well as in formulations prepared without antigen the protein contents was below the level of detection;
- By ELISA: the concentrations of the total formulation and the pellet were in the range of 0.6 - 1.2 mg/mL, while the supernatant concentration was 0.01-0.02

mg/mL. In the formulations prepared without antigen the protein concentration was below the detection limit of the method;

- By SDS-PAGE: The 29 kDa polypeptide shows no significant change after formulation into lipid aggregates.
- By Western blot: Trace amounts of 29 kDa polypeptide were found in the supernatant of the formulation manufactured with antigen.

After analysis the concentration of the centrifugated formulation was adjusted with 6mM Ca^{2+} buffer (vortex and 20 seconds in an ultrasonic bath) to give a composition in the formulation to be administered corresponding to 0.33 mg/mL of 29 kDa polypeptide. Administration of 300 µL would then give 100 µg administered 29 kDa polypeptide to each animal. Formulations manufactured without antigen were adjusted in the same way.

EXAMPLE 2: Analysis of the *H. pylori* 29 kDa protein for use as a vaccine, when used directly and when incorporated in a lipid aggregate formulation.

2.1. Materials & Methods

2.1.1. Animals

Female SPF BALB/c mice were purchased from Bomholt Breeding centre (Denmark). They were kept in ordinary makrolon cages with free supply of water and food. The animals were 4-6 weeks old at arrival.

2.1.2. Infection

After a minimum of one week of acclimatization, the animals were infected with a type 2 strain of *H. pylori* (strain 244, originally isolated from an ulcer patient). This strain has earlier proven to be a good colonizer of the mouse stomach. The bacteria were grown overnight in Brucella broth supplemented with 10% fetal calf serum, at

+37°C in a microaerophilic atmosphere (10% CO_2, 5% O_2). The animals were given an oral dose of omeprazole (400 µmol/kg) and after 3-5 h an oral inoculation of *H. pylori* (approximately 10^7-10^8 cfu/animal). Infection was checked in control animals 2-3 weeks after the inoculation.

2.1.3. Immunizations

The 7 groups of mice, with 6 animals/group were immunized 4 times over a 34 day period (day 1, 15, 25 and 35). Recombinant purified Helicobacter 29 kDa polypeptide was given at a dose of 100 µg/mouse either directly suspended in TES buffer or as incorporated into lipid aggregates (see above) . The groups are listed in Table I.

As an adjuvant, some groups of the animals were also given 10 µg/mouse of cholera toxin (CT) with each immunization. A total volume of 0.3 ml was given at each immunization. Omeprazole (400 µmol/kg) was given orally to the animals 3-5 h prior to immunization as a way of protecting the antigens from acid degradation. Animals were sacrificed 1-4 weeks after final immunization.

2.1.4. Analysis of infection

The mice were sacrificed by CO_2 and cervical dislocation. The abdomen was opened and the stomach removed. After cutting the stomach along the greater curvature, it was rinsed in saline. In in stomach half, an area of 25 mm^2 of the mucosa from the antrum and corpus was scraped separately with a surgical scalpel. The mucosa scraping was suspended in Brucella broth and plated onto Blood Skirrow plates. The plates were incubated under microaerophilic conditions for 3-5 days and the number of colonies was counted. The identity of *H. pylori* was ascertained by urease and catalase test and by direct microscopy or Gram staining.

2.1.5 Analysis of Immune response

Mucosal antibodies were collected by the following technique. One half of the rinsed stomach was placed mucosal side up on a piece of paper. Likewise the duodenum was cut open and placed mucosal side up. One standardized round filter paper (30.4 mm^2) was placed on the antrum and one on the corpus musosa. After 10 minutes the papers were transferred to one tube with 200µl special buffer containing protease inhibitors. A paperstrip 4.8x19 mm (91.2 mm^2) was placed in the same way on the duodenum mucosa and subsequently treated in the same way. The buffer solution was, after a minimum of one hour extraction of the filter papers, used directly for ELISA measurements of antibody concentration or keep frozen at -20°C.

Serum antibodies were collected from blood drawn by heart-puncture under - anesthesia. Prior to centrifugation, the blood was diluted with equal amount of PBS. The serum was kept at -20°C until analysis.

Mucosal antibodies were measured using an ELISA were 29 kDa polypeptide was plated followed by addition of mucosal extract. The response was developed by using Alkaline Phospahatase labelled anti-IgA and anti-Ig. Standad curves were created from plating known amounts of mouse IgA and Ig instead of the unknown sample. Serum antibodies were measured in a similar way but in this case both membrane proteins of the infecting strain i.e. 244 as well as 29 kDa polypeptide was plated.

2.2. Results

2.2.1. Eradication of infection

The animals in this study were infected with *H. pylori* strain 244 one month prior to immunizations. Mice in groups of six were then immunized with either cholera toxin (CT) or CT together with the *Helicobacter* 29 kDa polypeptide either directly or formulated in lipid aggregates (LA). Lipid aggregates were also tested without any

protein incorporation with and without CT (see Table I) Control animals received vehicle only (TES buffer). One week after the final immunization, the animals were sacrificed and colony forming units (CFU) was determined (see Fig. 1a, and Fig. 1b). All control animals, as well as those receiving CT, LA and CT+LA were heavily infected in both antrum and corpus. Animals actively immunized with 29 kDa polypeptide plus CT, had significantly decreased bacterial content (CFU values) compared with the controls. In 2/5 mice no bacteria could be detected in the corpus. When the 29 kDa polypeptide was formulated in lipid aggregates according to the present invention the degree of *H. pylori* colonization in the gastric mucosa decreased compared to administration of lipid aggregate alone. The addition of CT to 29 kDa polypeptide containing lipid aggregate further decreased the bacterial content, especially in the antrum.

2.2.2 Mucosal antibody response

Specific mucosal antibodies towards 29 kDa polypeptide of general Ig and of IgA class was measured. In the general Ig class increased levels were seen following immunization with HpaA+CT, in the group were 29 kDa polypeptide was formulated with lipid aggregates and when CT was added to the latter preparation (see Figure 2). The appearence of specific IgA showed the same pattern (see Figure 3).

2.2.3 Serum antibody response

Serum antibody response was measured towards a membrane prepartion of *H. pylori* strain 244 (the strain used for infection of the mice) and towards 29 kDa polypeptide. All animals had high titers against 244. No difference between the groups could be detected (see Figure 4). This response is mainly seen as a sign of appropriate infection with *H. pylori*. The specific serum response to 29 kDa polypeptide preparation as a sign of successful presentation of the 29 kDa polypeptide to the systemic immune system was highest in the group with 29 kDa + CT followed by 29 kDa polypeptide

formulated with LA + CT. Also the lipid aggregate formulation of 29 kDa polypeptide without CT gave an increased number of serum antibodies (see Fig. 5).

2.3. Discussion and Applicability of the invention

It has previously been shown that the administration of 29 kDa + CT can give rise to an immune response measured as mucosal and systemic antibodies, which is capable of eradicate of decrease the number of *H. pylori* associated with the mouse gastric mucosa. In the absence of the adjuvant CT, no effect has been obtained.

Therefore, surprisingly it was found that 29 kDa polypeptide formulated in the lipid aggregates according to the present invention could, even in the absence of CT, induce an immune response of such nature that the degree of *H. pylori* colonization of the gastric mucosa of mice decreased.

2.4. Conclusions

- The *H. pylori* surface located protein 29 kDa polypeptide has been shown to be a strong and consistent antigen when in a purified recombinant form is presented to a mucosal surface in a physiological buffer solution.
- The 29 kDa polypeptide will stimulate a competent local immune response capable of eradicating *H. pylori* colonizing the gastric mucosa.
- Repeated administration of 29 kDa polypeptide during weeks or months will potentiate the effect.
- The effect of 29 kDa polypeptide has been dependent on the simultaneous presence of the mucosal adjuvant Cholera toxin (CT).
- Surprisingly 29 kDa polypeptide will induce an adequate local immune response even in the absence of CT, when it is formulated in lipid aggregates.

It is concluded that 29 kDa polypeptide can evoke a relevant immune response locally in the gastric mucosa both when administered together with cholera toxin as well as when it is formulated in a lipid aggregate.

This lipid aggregate formulation obviously has the capability to present *H. pylori* antigens to the local immune system in such a way that an adequate eradicating immune response is obtained.

TABLE I: Therapeutic immunisation groups

Substance:	Dose:	Administration	Number of doses
1. Control, vehichle	0,3 ml/dose	p.o	4
2. Cholera toxin (CT) 10µg	0,3 ml/dos	p.o	4
3. Lipid aggregates (LA)	0,3 ml/dose	p.o	4
4. LA + CT	0,3 ml/dose	p.o	4
5. 29 kDa(100µg) + CT	0,3 ml/dose	p.o	4
6. LA(29 kDa) 100 µg	0,3 ml/dose	p.o	4
7. LA(29 kDa) + CT	0,3 ml/dose	p.o	4

Further support is found in the accompaning figures as discussed above.

A suitable antigen for the formulation according to the present invention is the 29 kDa polypeptide described in the Applicant's co-pending WO96/38475. The 29 kDa polypeptide used in examples 1 and 2 was prepared according to the published WO96/38475.

DEPOSITION OF MICROORGANISMS

The plasmid pAE1 has been deposited under the Budapest Treaty at the National Collections of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland, UK, and under accession number NCIMB 40732. The date of deposit is 16 May 1995.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: ASTRA AB

(B) STREET: Västra Mälarehamnen 9

(C) CITY: Södertälje

(E) COUNTRY: Sweden

(F) POSTAL CODE (ZIP): S-151 85

(G) TELEPHONE: +46 8 553 260 00

(H) TELEFAX: +46 8 553 288 20

(I) TELEX: 19237 astra s

(ii) TITLE OF INVENTION: New Pharmaceutical Formulation of Polypeptides

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1670 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:793..1575

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION:793..1572

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GATCCTATCG CGCCAAAGGT GGTATTAGGA ATAAGAGCTT GATTATTAAT CTCCCTGGTA 60

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GATTTGATTT TAGGGAATTA CATGCAAGTG AATGAAAAAA ACATTCAAGC GTTTGCCCCC 180

AAACAATAAG GTAAAAAATG CCACTCACTC ATTTGAATGA AGAAAATCAA CCTAAAATGG 240

TGGATATAGG GGATAAAGAA ACCACTGAAA GAATCGCTCT AGCAAGCGGT CGTATCAGCA 300

TGAATAAAGA GGCTTATGAC GCTATTATCA ATCATGGCGT CAAAAAGGGT CCGGTATTAC 360

AAACTGCTAT TATTGCTGGG ATTATGGGGG CTAAAAAGAC AAGCGAACTC ATTCCCATGT 420

GCCATCCAAT CATGCTCAAT GGGGTGGATA TTGATATTTT AGAAGAAAAA GAGACTTGTA 480

GTTTTAAACT CTATGCGAGA GTCAAAACTC AAGCTAAAAC GGGCGTAGAA ATGGAAGCGC 540

TAATGAGTGT GAGCGTAGGG CTTTTAACCA TTTATGACAT GGTGAAAGCC ATTGATAAGA 600

GCATGACAAT TAGCGGTGTG ATGCTGGAAT ATAAAAGTGG AGGCAAAAGT GGGGATTATA 660

ACGCTAAAAA ATAGAAAAAG ACTGATAATC TAAAGATATT AGGGTAAAAT AACATTTTGA 720

CAACAAAAGC GTGTTGGTTG CTTCGGATTT GTTGTTATAG AAGTCTAAAA TATTACAATC 780

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1 5 10

AAA AAA TGC CTT TTA GGC GCG AGC GTG GTG GCT TTA TTA GTG GGA TGC 876
Lys Lys Cys Leu Leu Gly Ala Ser Val Val Ala Leu Leu Val Gly Cys
15 20 25

AGC CCG CAT ATT ATT GAA ACC AAT GAA GTC GCT TTG AAA TTG AAT TAC 924
Ser Pro His Ile Ile Glu Thr Asn Glu Val Ala Leu Lys Leu Asn Tyr

30 35 40

CAT CCA GCT AGC GAG AAA GTT CAA GCG TTA GAT GAA AAG ATT TTG CTT 972
His Pro Ala Ser Glu Lys Val Gln Ala Leu Asp Glu Lys Ile Leu Leu
45 50 55 60

TTA AGG CCA GCT TTC CAA TAT AGC GAT AAT ATC GCT AAA GAG TAT GAA 1020
Leu Arg Pro Ala Phe Gln Tyr Ser Asp Asn Ile Ala Lys Glu Tyr Glu
65 70 75

AAC AAA TTC AAG AAT CAA ACC GCG CTC AAG GTT GAA CAG ATT TTG CAA 1068
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Gly Glu Ile Val Leu Arg Pro Asp Pro Lys Arg Thr Ile Gln Lys Lys
125 130 135 140

TCA GAA CCC GGG TTA TTA TTC TCC ACC GGT TTG GAC AAA ATG GAA GGG 1260
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145 150 155

GTT TTA ATC CCG GCT GGG TTT ATT AAG GTT ACC ATA CTA GAG CCT ATG 1308
Val Leu Ile Pro Ala Gly Phe Ile Lys Val Thr Ile Leu Glu Pro Met
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AGT GGG GAA TCT TTG GAT TCT TTT ACG ATG GAT TTG AGC GAG TTG GAC 1356
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175 180 185

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190 195 200

TTA GTT AGC ACT ATG GTT AAG GGA ACG GAT AAT TCT AAT GAC GCG ATC 1452
Leu Val Ser Thr Met Val Lys Gly Thr Asp Asn Ser Asn Asp Ala Ile
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225 230 235

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GAA TTA AAA GGC AAA AGA AAC CGA TAA AAACAAATAA CGCATAAGAA 1595
Glu Leu Lys Gly Lys Arg Asn Arg *

255 260

AAGAACGCTT GAATAAACTG CTTAAAAAGG GTTTTTTAGC GTTCTTTTTG AGCGTGTATT 1655

TAAGGGCTGA TGATC 1670

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 261 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Arg Ala Asn Asn His Phe Lys Asp Phe Ala Trp Lys Lys Cys Leu
1 5 10 15

Leu Gly Ala Ser Val Val Ala Leu Leu Val Gly Cys Ser Pro His Ile
20 25 30

Ile Glu Thr Asn Glu Val Ala Leu Lys Leu Asn Tyr His Pro Ala Ser
35 40 45

Glu Lys Val Gln Ala Leu Asp Glu Lys Ile Leu Leu Leu Arg Pro Ala
50 55 60

Phe Gln Tyr Ser Asp Asn Ile Ala Lys Glu Tyr Glu Asn Lys Phe Lys
65 70 75 80

Asn Gln Thr Ala Leu Lys Val Glu Gln Ile Leu Gln Asn Gln Gly Tyr
85 90 95

Lys Val Ile Ser Val Asp Ser Ser Asp Lys Asp Asp Phe Ser Phe Ala
100 105 110

Gln Lys Lys Glu Gly Tyr Leu Ala Val Ala Met Asn Gly Glu Ile Val
115 120 125

Leu Arg Pro Asp Pro Lys Arg Thr Ile Gln Lys Lys Ser Glu Pro Gly
130 135 140

Leu Leu Phe Ser Thr Gly Leu Asp Lys Met Glu Gly Val Leu Ile Pro
145 150 155 160

Ala Gly Phe Ile Lys Val Thr Ile Leu Glu Pro Met Ser Gly Glu Ser
165 170 175

Leu Asp Ser Phe Thr Met Asp Leu Ser Glu Leu Asp Ile Gln Glu Lys
180 185 190

Phe Leu Lys Thr Thr His Ser Ser His Ser Gly Gly Leu Val Ser Thr
195 200 205

Met Val Lys Gly Thr Asp Asn Ser Asn Asp Ala Ile Lys Ser Ala Leu
210 215 220

Asn Lys Ile Phe Ala Asn Ile Met Gln Glu Ile Asp Lys Lys Leu Thr
225 230 235 240

Gln Lys Asn Leu Glu Ser Tyr Gln Lys Asp Ala Lys Glu Leu Lys Gly
245 250 255

Lys Arg Asn Arg *
260

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1670 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION:793..1575

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION:793..1572

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GATCCTATCG CGCCAAAGGT GGTATTAGGA ATAAGAGCTT GATTATTAAT CTCCCTGGTA 60

AGTCCAAAAA GTATTAGAGA ATGCTTAGAG GCGGTTTTTC CAGCGATTCC TTATTGCGTG 120

GATTTGATTT TAGGGAATTA CATGCAAGTG AATGAAAAAA ACATTCAAGC GTTTGCCCCC 180

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TGGATATAGG GGATAAAGAA ACCACTGAAA GAATCGCTCT AGCAAGCGGT CGTATCAGCA 300

TGAATAAAGA GGCTTATGAC GCTATTATCA ATCATGGCGT CAAAAAGGGT CCGGTATTAC 360

AAACTGCTAT TATTGCTGGG ATTATGGGGG CTAAAAAGAC AAGCGAACTC ATTCCCATGT 420

GCCATCCAAT CATGCTCAAT GGGGTGGATA TTGATATTTT AGAAGAAAAA GAGACTTGTA 480

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TAATGAGTGT GAGCGTAGGG CTTTTAACCA TTTATGACAT GGTGAAAGCC ATTGATAAGA 600

GCATGACAAT TAGCGGTGTG ATGCTGGAAT ATAAAAGTGG AGGCAAAAGT GGGGATTATA 660

ACGCTAAAAA ATAGAAAAAG ACTGATAATC TAAAGATATT AGGGTAAAAT AACATTTTGA 720

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Ser Pro His Ile Ile Glu Thr Asn Glu Val Ala Leu Lys Leu Asn Tyr
30 35 40

CAT CCA GCT AGC GAG AAA GTT CAA GCG TTA GAT GAA AAG ATT TTG CTT 972
His Pro Ala Ser Glu Lys Val Gln Ala Leu Asp Glu Lys Ile Leu Leu
45 50 55 60

TTA AGG CCA GCT TTC CAA TAT AGC GAT AAT ATC GCT AAA GAG TAT GAA 1020
Leu Arg Pro Ala Phe Gln Tyr Ser Asp Asn Ile Ala Lys Glu Tyr Glu
65 70 75

AAC AAA TTC AAG AAT CAA ACC GCG CTC AAG GTT GAA CAG ATT TTG CAA 1068
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95 100 105

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145 150 155

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160 165 170

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175 180 185

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Ile Gln Glu Lys Phe Leu Lys Thr Thr His Ser Ser His Ser Gly Gly
190 195 200

```
TTA GTT AGC ACT ATG GTT AAG GGA ACG GAT AAT TCT AAT GAC GCG ATC      1452
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205                 210                 215                 220

AAG AGA GCT TTG AAT AAG ATT TTT GCA AAT ATC ATG CAA GAA ATA GAC      1500
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                225                 230                 235

AAA AAA CTC ACT CAA AAG AAT TTA GAA TCT TAT CAA AAA GAC GCC AAA      1548
Lys Lys Leu Thr Gln Lys Asn Leu Glu Ser Tyr Gln Lys Asp Ala Lys
            240                 245                 250

GAA TTA AAA GGC AAA AGA AAC CGA TAA AAACAAATAA CGCATAAGAA            1595
Glu Leu Lys Gly Lys Arg Asn Arg  *
        255                 260

AAGAACGCTT GAATAAACTG CTTAAAAAGG GTTTTTTAGC GTTCTTTTTG AGCGTGTATT    1655

TAAGGGCTGA TGATC                                                    1670
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 261 amino acids

(B) TYPE: amino acid

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Met Arg Ala Asn Asn His Phe Lys Asp Phe Ala Trp Lys Lys Cys Leu
1 5 10 15

Leu Gly Ala Ser Val Val Ala Leu Leu Val Gly Cys Ser Pro His Ile
20 25 30

Ile Glu Thr Asn Glu Val Ala Leu Lys Leu Asn Tyr His Pro Ala Ser
35 40 45

Glu Lys Val Gln Ala Leu Asp Glu Lys Ile Leu Leu Leu Arg Pro Ala
50 55 60

Phe Gln Tyr Ser Asp Asn Ile Ala Lys Glu Tyr Glu Asn Lys Phe Lys
65 70 75 80

Asn Gln Thr Ala Leu Lys Val Glu Gln Ile Leu Gln Asn Gln Gly Tyr
85 90 95

Lys Val Ile Ser Val Asp Ser Ser Asp Lys Asp Asp Phe Ser Phe Ala
100 105 110

Gln Lys Lys Glu Gly Tyr Leu Ala Val Ala Met Asn Gly Glu Ile Val
115 120 125

Leu Arg Pro Asp Pro Lys Arg Thr Ile Gln Lys Lys Ser Glu Pro Gly

130 135 140

Leu Leu Phe Ser Thr Gly Leu Asp Lys Met Glu Gly Val Leu Ile Pro
145 150 155 160

Ala Gly Phe Ile Lys Val Thr Ile Leu Glu Pro Met Ser Gly Glu Ser
165 170 175

Leu Asp Ser Phe Thr Met Asp Leu Ser Glu Leu Asp Ile Gln Glu Lys
180 185 190

Phe Leu Lys Thr Thr His Ser Ser His Ser Gly Gly Leu Val Ser Thr
195 200 205

Met Val Lys Gly Thr Asp Asn Ser Asn Asp Ala Ile Lys Arg Ala Leu
210 215 220

Asn Lys Ile Phe Ala Asn Ile Met Gln Glu Ile Asp Lys Lys Leu Thr
225 230 235 240

Gln Lys Asn Leu Glu Ser Tyr Gln Lys Asp Ala Lys Glu Leu Lys Gly
245 250 255

Lys Arg Asn Arg *
260

Legends to the figures:

Fig.1a: Number of *Heliocobacter pylori* (as CFU, geometric mean values) in 25mm^2 scrapings of antrum and corpus, following oral administration of different agents.
CT=Cholera toxin; LA=Lipid aggregates; LA(29kDa)=29 kDa formulated in Lipid aggregates. n=6 for all animals, except CT (n=3) and 29kDa + CT (n=5).

Fig.1b: As 1a) but displayes as total number of *Heliocobacter pylori* in the stomach. Abbrevations see: Fig.1a)

Fig.2: Total amount of mucosal immunoglobulin (Ig) with a specificity for 29kDa polypeptide, detected by ELISA technique. Abbrevations see: Fig.1a

Fig.3: Amount of 29kDa specific mucosal IgA antibodies. Abbrevations see: Fig.1a

Fig.4: *Heliocobacter* specific IgG antibodies in sera. Abbrevations see: Fig.1a

Fig.5: 29kDa specific IgG antibodies in sera. Abbrevations see: Fig.1a

CLAIMS

1. A pharmaceutical formulation comprising a lipid aggregate of a negatively charged lipid component or a lipid mixture and at least one antigenic polypeptide which is a *Helicobacter pylori* antigen or nucleic acid molecules coding for such polypeptides, and optionally a pharmaceutically acceptable carrier.

2. A pharmaceutical formulation according to claim 1, wherein the antigenic polypeptide is a recombinant polypeptide.

3. A pharmaceutical formulation according to claim 2, wherein the recombinant polypeptide is a hydrophobic *H. pylori* antigen.

4. A pharmaceutical formulation according to claim 3, wherein the antigen is a *H. pylori* membrane protein.

5. A pharmaceutical formulation according to claim 4, wherein the antigen is a polypeptide which has an amino acid sequence identical with, or substantially similar to, a *H. pylori* surface-exposed antigen with an approximate molecular weight of 29 kDa.

6. A pharmaceutical formulation according to claim 5, wherein the polypeptide has an amino acid sequence identical with, or substantially similar to, SEQ ID NO: 2 in the Sequence Listing.

7. A pharmaceutical formulation according to claim 1, wherein the polypeptide is a peptide with a lenght of at least 5 amino acids comprising an immunogenic epitope of a polypeptide which is a Helicobacter pylori membrane protein.

8. A pharmaceutical formulation according to claim 1, wherein the polypeptide is replaced by an isolated and purified nucleic acid molecule which has a nucleotide sequence coding for a polypeptide according to claim 5 or claim 6.

9. A pharmaceutical formulation according to claim 1, wherein the lipid aggregate in addition to the antigenic *H. pylori* polypeptide comprises a negatively charged lipid component or a lipid mixture comprises a cation component.

10. A pharmaceutical formulation according to claim 9, wherein the negatively charged lipid component is a phospholipid.

11. A pharmaceutical formulation according to claim 10, wherein the phospholipid is selected from the group consiting of phosphatidylserine, phosphatidylinositol, phosphatidic acid and phosphatidylglycerol.

12. A pharmaceutical formulation according to any of claims 9, 10 or 11, wherein there is also present a cation component which chelates or complexes with the negatively charged lipid component.

13. A pharmaceutical formulation according to claim 12, wherein the cation component is selected from the group consisting of Ca^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Mg^{2+} and Al^{3+}.

14. A pharmaceutical formulation according to claim 1, wherein the formulation in addition to the lipid aggregate comprises a vaccine adjuvant.

15. A pharmaceutical formulation according to claim 1, for use as a therapeutic vaccine in a mammal, including man, which is infected by *Helicobacter pylori.*

16. A pharmaceutical formulation according to claim 1, for use as a prophylactic vaccine in a mammal, including man, from infection by *Helicobacter pylori.*

17. A pharmaceutical formulation according to claim 1, wherein the formulation is delivered to a mucosal surface by oral, parental, rectal or nasal administration.

18. A process for the manufacture of a pharmaceutical formulation comprising a lipid aggregate of a negatively charged lipid component or a lipid mixture and at least one antigenic polypeptide which is *H. pylori* antigen, comprising the steps of

i) formation of a lipid film composed of the negatively charged lipid component or lipid mixture, optionally with other lipids,
ii) addition of the *H. pylori* antigen in a surfactant solution
iii) exchange the surfactant with a positively charged ion.

19. A process according to claim 18, wherein step iii) is carried out by dialysis in one step, or in two steps.

20. Use of a formulation according to anyone of claims 1 to 18 in the manufacture of a formulation for the treatment or protection of *H. pylori* infections.

21. Use of a formulation according to anyone of claims 1 to 18 in the manufacture of a vaccine eliciting a protective immune response against *H. pylori* infections.

22. A method of eliciting in a mammal a protective immune response against *H. pylori* infections, comprising the step of administering to the said mammal an immunogenically effective amount of a pharmaceutical formulation according to any of claims 1 to 18.

23. A method according to claim 22, wherein the administration is carried out by an oral, parenteral, rectal or nasal route.

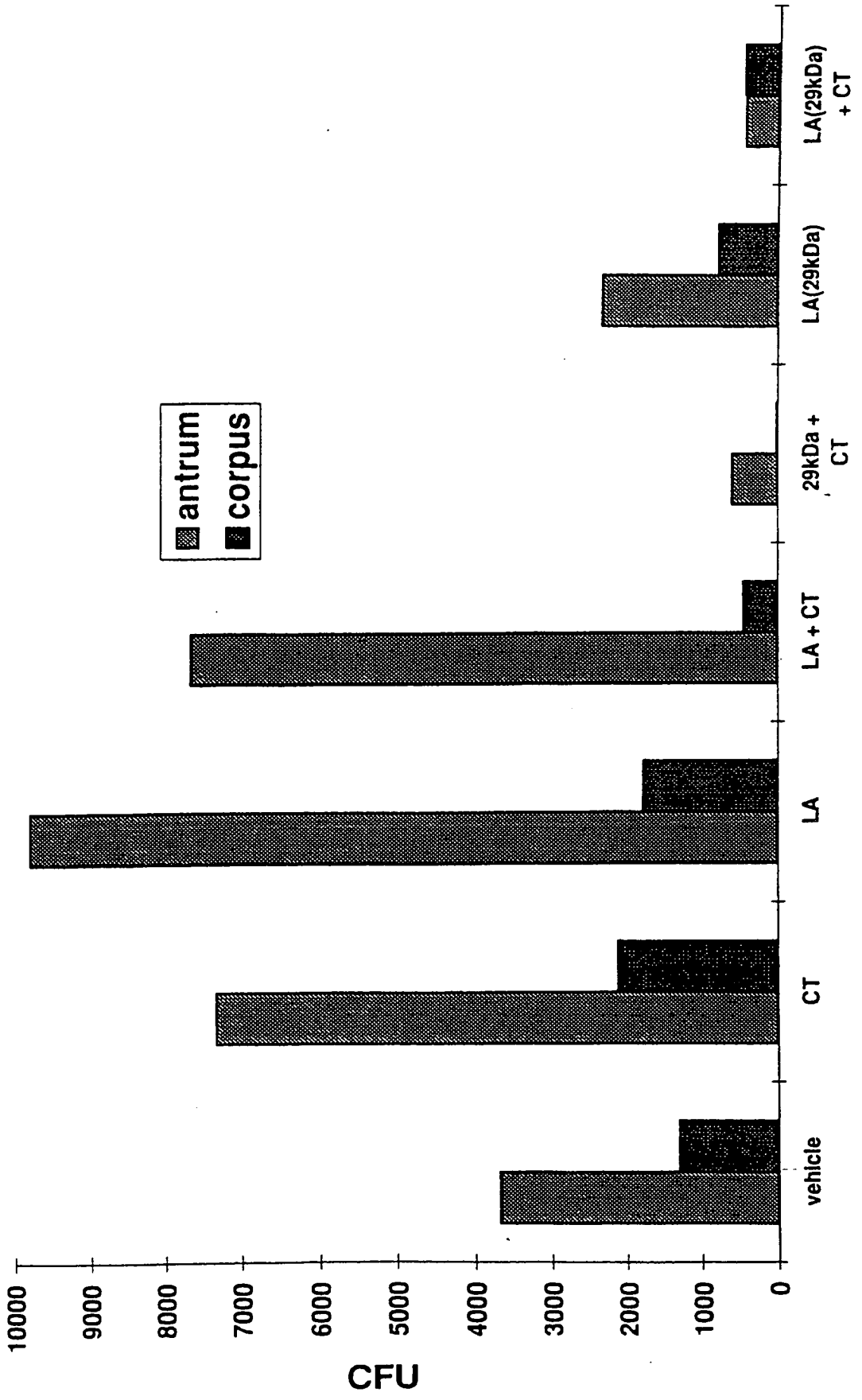

Fig. 1a

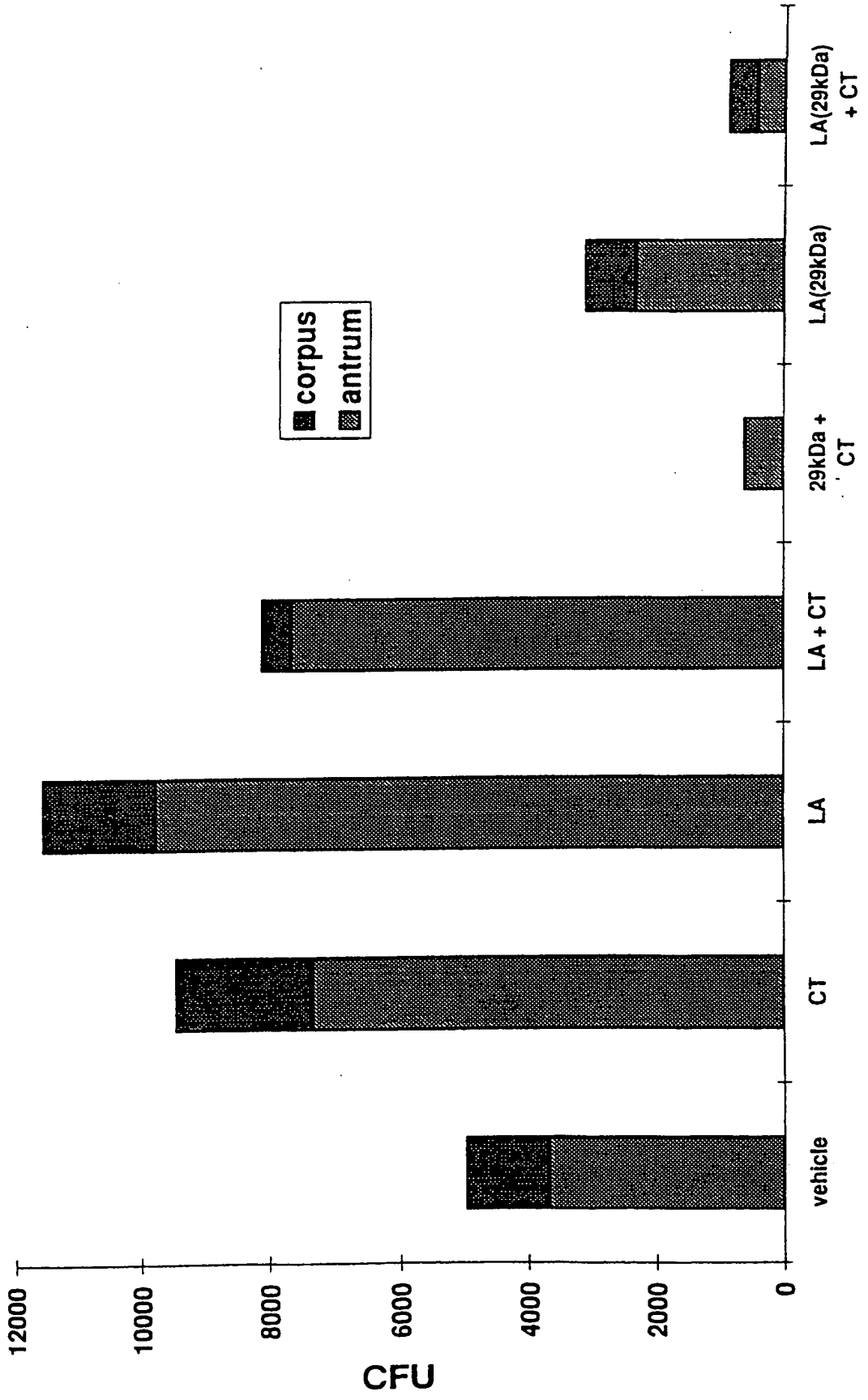
corpus
antrum
vehicle
CT
LA
LA + CT
29kDa + CT
LA(29kDa)
LA(29kDa) + CT
0
2000
4000
6000
8000
10000
12000
CFU

Fig. 1b

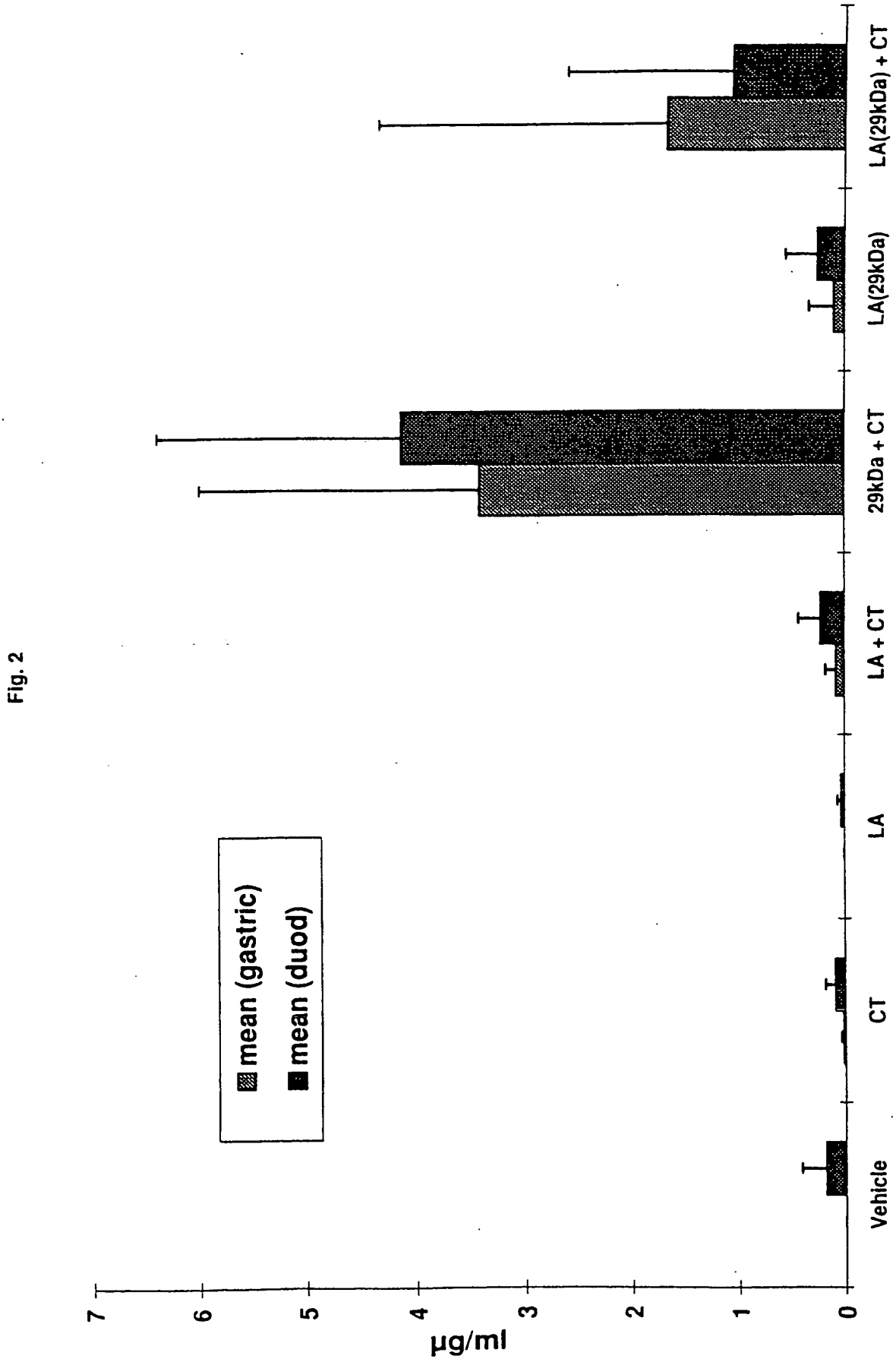

Fig. 2

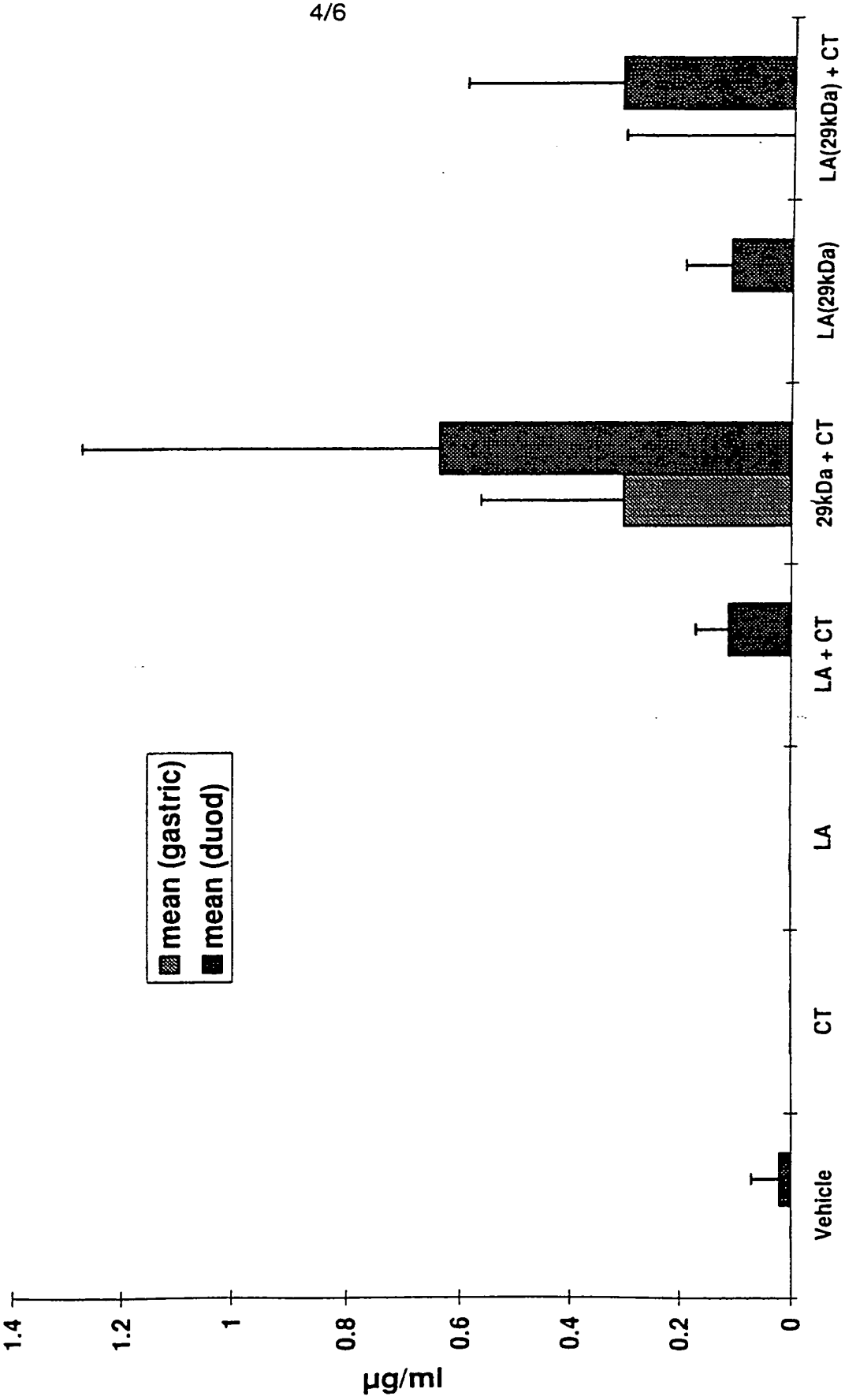
mean (gastric)
mean (duod)
µg/ml
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Vehicle
CT
LA
LA + CT
29kDa + CT
LA(29kDa)
LA(29kDa) + CT

Fig. 3

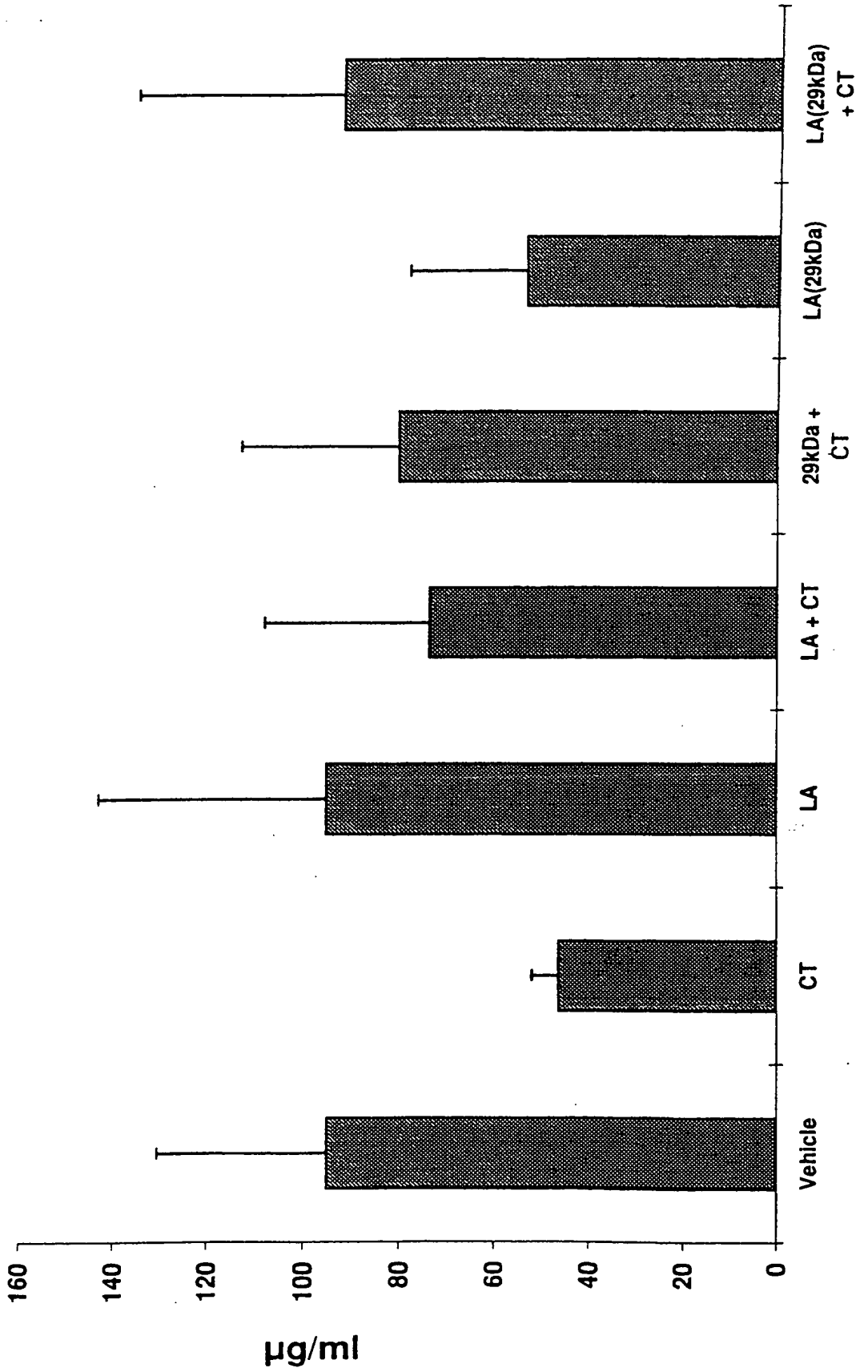

Fig. 4

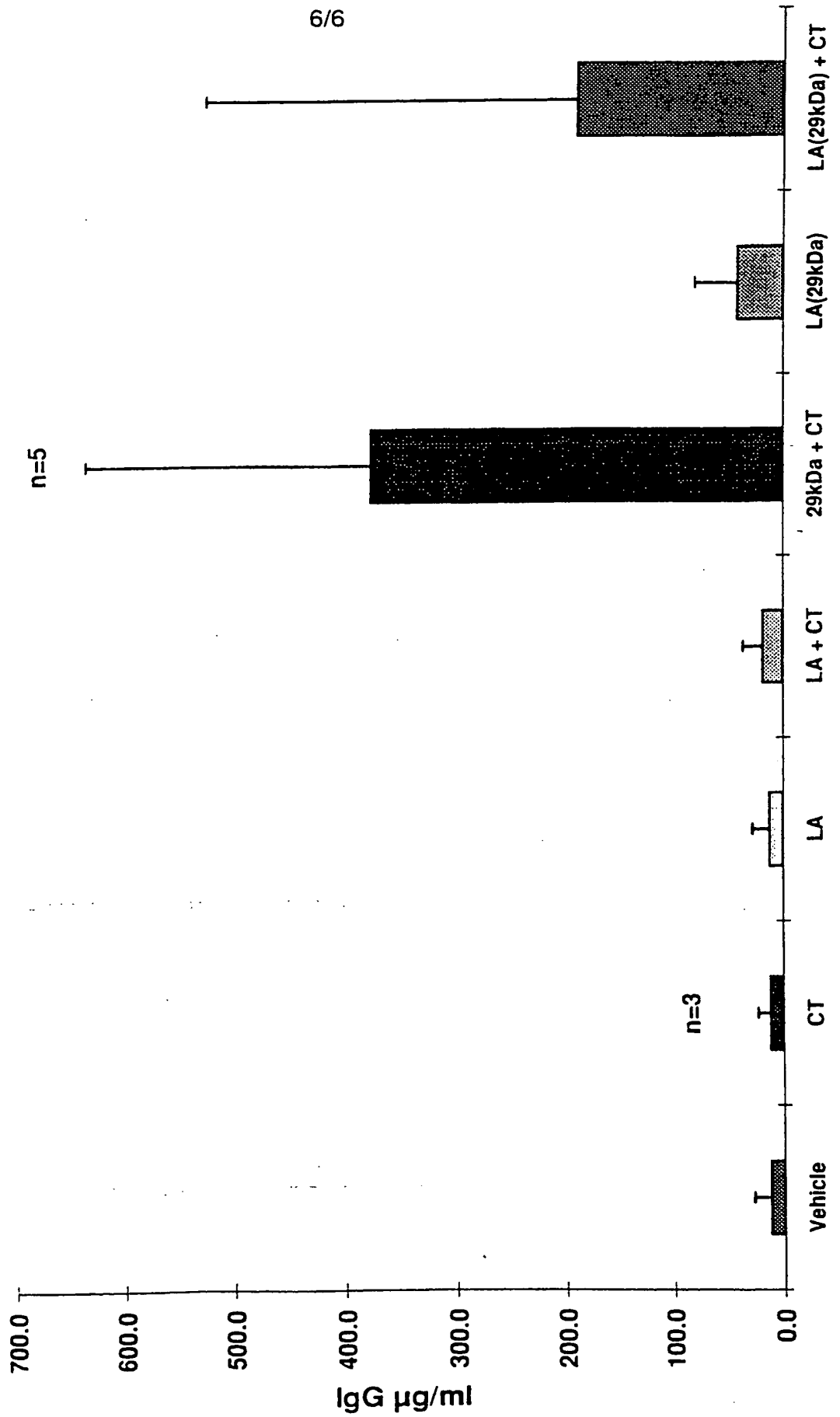

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01927

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 39/106, A61K 9/127 // C07K 14/205
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K, C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, SCISEARCH, CA, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Microbiology, Volume 141, 1995, Catherine J. Luke et al, "Identification of a 29 kDa flagellar sheath protein in Helicobacter pylori using a murine monoclonal antibody" page 597 - page 604 --	1-23
Y	WO 9509648 A1 (GOULD-FOGERITE, SUSAN), 13 April 1995 (13.04.95) --	1-23
Y	EMBL, Databas GenBank/DDBJ, accession no. X92502, Jones, A.C. et al: "Gene Cloning of flagellar sheat protein of Helicobacter pylori"; & J. Bacteriol. 175 (3), 674-683 (1993) --	6

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 February 1998	04 -03- 1998
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 97/01927

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Gut, Volume 37, No 1, 1995, A Jones et al, "Gene cloning of a flagellar sheath protein of Helicobacter Pylori", page A63 -- --------	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

03/02/98

International application No.
PCT/SE 97/01927

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9509648 A1	13/04/95	AU 7959094 A	01/05/95
		CA 2169297 A	13/04/95
		EP 0722338 A	24/07/96
		US 5643574 A	01/07/97

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